The transcription factor JunD mediates transforming growth factor beta-induced fibroblast activation and fibrosis in systemic sclerosis

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Abstract: OBJECTIVES: Transforming growth factor (TGF) has been identified as a key player in fibrotic diseases. However, the molecular mechanisms by which TGF activates fibroblasts are incompletely understood. Here, the role of JunD, a member of the activator protein 1 (AP-1) family of transcription factors, as a downstream mediator of TGF signalling in systemic sclerosis (SSc), was investigated. METHODS: The expression of JunD was analysed by real-time PCR, immunofluorescence, western blotting and immunohistochemistry. The canonical Smad pathway was specifically targeted by small interfering (si)RNA. The expression of extracellular matrix proteins in JunD deficient (JunD(-/-)) fibroblasts was analysed by real-time PCR and hydroxyproline assays. The mouse model of bleomycin-induced dermal fibrosis was used to assess the role of JunD in experimental fibrosis. RESULTS: JunD was overexpressed in SSc skin and in cultured fibroblasts in a TGF dependent manner. The expression of JunD colocalised with pSmad 3 in fibrotic skin and silencing of Smad 3 or Smad 4 by siRNA prevented the induction of JunD by TGF . JunD(-/-) fibroblasts were less responsive to TGF and released less collagen upon stimulation with TGF . Moreover, JunD(-/-) mice were protected from bleomycin-induced fibrosis with reduced dermal thickening, decreased myofibroblast counts and lower collagen content of lesional skin. CONCLUSIONS: These data demonstrate that JunD is overexpressed in SSc and that JunD is a mediator of the profibrotic effects of TGF . Considering that inhibitors of AP-1 signalling have recently been developed and are available for clinical trials in SSc, these findings may have translational implications.

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The transcription factor JunD mediates transforming growth factor-β induced fibroblast activation and fibrosis in systemic sclerosis

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ABSTRACT

Objectives. Transforming growth factor-β (TGF-β) has been identified as a key-player in fibrotic diseases. However, the molecular mechanisms by which TGF-β activates fibroblasts are incompletely understood. Here, we investigated the role of JunD, a member of the AP-1 family of transcription factors, as a downstream mediator of TGF-β signaling in SSc.

Methods. The expression of JunD was analyzed by real-time PCR, immunofluorescence, Western blot and immunohistochemistry. Canonical Smad pathway was specifically targeted by siRNA. The expression of extracellular matrix proteins in JunD deficient ($JunD^{-/-}$) fibroblasts was analyzed by real-time PCR and hydroxyproline assays. The mouse model of bleomycin-induced dermal fibrosis was used to assess the role of JunD in experimental fibrosis.

Results. JunD was overexpressed in SSc skin and in cultured fibroblasts in a TGF-β dependent manner. The expression of JunD co-localized with pSmad 3 in fibrotic skin and silencing of Smad 3 or Smad 4 by siRNA prevented the induction of JunD by TGF-β. $JunD^{-/-}$ fibroblasts were less responsive to TGF-β and released less collagen upon stimulation with TGF-β. Moreover, $JunD^{-/-}$ mice were protected from bleomycin induced fibrosis with reduced dermal thickening, decreased myofibroblast counts and lower collagen content of lesional skin.

Conclusions. These data demonstrate that JunD is overexpressed in SSc and that JunD is a mediator of the pro-fibrotic effects of TGF-β. Considering that inhibitors of AP-1 signaling have recently been developed and would be available for clinical trials in SSc, our findings might have translational implications.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune connective tissue disease that affects the skin and a variety of internal organs including the lungs, heart and gastrointestinal tract. The histopathological hallmarks of early stages of SSc are perivascular inflammatory infiltrates and a reduced capillary density [1-3]. Later stages are dominated by an excessive accumulation of extracellular matrix components, which is caused by a pathologic activation of fibroblasts [4]. Transforming growth factor-β (TGF-β) has been identified as a central mediator of fibroblast activation in SSc [4]. However, the intracellular signaling cascades, by which this cytokine stimulates the production of extracellular matrix, are only partially understood, and the current knowledge has not yet been translated into anti-fibrotic therapies for clinical use [5-7].

JunD belongs to the Jun (c-Jun, JunB) family, which either forms homodimers or heterodimers with members of the Fos (c-Fos, FosB, Fos-related antigen (Fra)-1 and Fra-2) family to regulate the transcription of target genes {Jochum, #17655;Wagner, #17656}. Jun and Fos proteins are also summarized as activator protein-1 (AP-1) transcription factors. The capability to activate or suppress gene expression depends on the composition of the AP-1 complex, because the different dimers vary significantly in their DNA binding affinity [8]. Mice lacking JunD are viable and, apart from male sterility, develop normally [9]. However, deficiency of JunD impairs stress responses. Thus, fibroblasts deficient for JunD are particular susceptible to stress-induced apoptosis [10]. In addition, mice lacking JunD show increased and progressive kidney damage after subtotal nephrectomy [11] and demonstrate increased cardiomyocyte apoptosis and mortality upon pressure overload [12]. Consistent with a role of JunD in stress responses and cellular activation, JunD is prominently induced in hepatic stellate cells upon activation [13]. The induction of JunD is more pronounced than for any other member of the Jun family suggesting that JunD is of particular importance for the

activation of hepatic stellate cells. Considering the prominent role of hepatic stellate cells in liver fibrosis [14-16], we hypothesized that JunD might also regulate the activation of fibroblasts and contribute to tissue fibrosis in SSc.

MATERIAL AND METHODS

Patients and fibroblast cultures

Fibroblast cultures were obtained from lesional skin biopsies of 20 SSc patients and 12 healthy volunteers matched for age and sex. All SSc patients fulfilled the criteria ACR criteria for SSc. The median age of SSc patients was 53 years (range: 32 -78 years) and their median disease duration was 6 years (range: 0.5 - 13 years); All patients had diffuse cutaneous SSc and positive antinuclear antibodies; none of the patients was positive for anti-centromere antibodies, but 10 were positive for anti-topoisomerase-1 antibodies. None of the patients was treated with immunosuppressive or other potentially disease modifying drugs at the time of biopsy. 15 biopsies were taken from involved skin of the mid volar surface of the forearm (15±2 cm proximal to the ulna styloid). 5 additional biopsies were obtained from the non-involved skin of the upper arm. All patients and controls signed a consent form approved by the local institutional review boards.

Fibroblast cultures were obtained from SSc patients and healthy volunteers and from mice deficient for JunD ($JunD^{-/-}$) and wildtype littermates ($JunD^{+/+}$) were cultured in Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 containing 10 % heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 2.5 µg/ml amphotericin B (all from Invitrogen, Karlsruhe, Germany). In selected experiments, fibroblasts were stimulated with recombinant TGF-β in a concentration of 10 ng/ml, respectively (R&D Systems, Wiesbaden-Nordenstadt, Germany). This latter concentration represents the standard concentration used for the stimulation of dermal fibroblasts [17-18]. Fibroblasts from passages 4–8 were used for the experiments.

Western blot analysis

For Western blot, 10 µg of protein in Laemmli sample loading buffer were separated on 10% SDS-polyacrylamide gel and electro transferred onto PVDF membranes. After

blocking with 5 % non-fat dry milk for 1 h, blots were incubated with specific polyclonal primary antibodies (1:400) diluted in 2 % non-fat dry milk overnight at 4 °C. For the detection of JunD, polyclonal antibodies against JunD (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) were used. Signals were visualized with an ECL Plus™ Western Blotting Detection System and exposed to high performance chemiluminescence film.

Immunofluorescence staining

Fibroblasts were incubated for 24 h with 0.1 % DMEM/F12 and stimulated for 1 h, 3 h, 6 h, 9 h, 24 h with 10 ng/ml of TGF-β. The cultured fibroblasts were first washed with 1x PBS and then fixed with 4 % methanol free paraformaldehyde at room temperature for 10 min. Fixed cells were permeabilized for 5 min at room temperature with 0.25 % Triton X-100. Next, cells were incubated with 5 % (v/v) horse serum blocking solution for 1 h. JunD was detected by incubation with polyclonal rabbit anti-human JunD antibodies over night at 4 °C. Irrelevant isotype antibodies were used as controls. Polyclonal goat anti-rabbit antibodies labeled with fluorescent dye Alexa Fluor 488 were used as secondary antibodies. In addition, cell nuclei were stained with DAPI. Images were captured at a 200-fold magnification.

Bleomycin-induced dermal fibrosis in JunD deficient mice

$JunD^{-/-}$ mice on a C57/Bl6 background have been described before [9]. Wildtype C57/Bl6 ($JunD^{+/+}$) littermates were used as controls. Skin fibrosis was induced in 6-week-old mice by local injections of bleomycin every other day for 4 weeks [19]. 100 µl of bleomycin dissolved in 0.9 % sodium chloride (NaCl) at a concentration of 0.5 mg/ml was administered by subcutaneous injections in defined areas of 1 cm^2 at the upper back. Subcutaneous injections of 100 µl 0.9 % NaCl were used as controls. One group of JunD $^{-/-}$ mice and one group of JunD $^{+/+}$ mice were challenged with bleomycin, whereas the remaining two groups were injected with NaCl. After 4 weeks, mice were sacrificed to analyze the dermal thickness,

the hydroxyproline content as well as the number of myofibroblasts in lesional skin. 20 JunD $^{-/-}$ mice and 16 JunD $^{+/+}$ littermates were used for these analyses.

Evaluation of dermal thickness

Lesional skin areas were excised, fixed in 4 % formalin for 6 h and embedded in paraffin. 5 µm sections were cut and stained with hematoxylin and eosin. The dermal thickness was measured at 100-fold magnification by measuring the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction at three sites from lesional skin of each mouse [18]. The analysis was performed in a blinded manner by two independent examiners.

Hydroxyproline assay

The amount of collagen protein in skin samples was determined via hydroxyproline assay [20]. After digestion of punch biopsies (Ø 3mm) in 6 M HCl for three hours at 120 °C, the pH of the samples was adjusted to 6 with 6 M NaOH. Afterwards, 0.06 M chloramine T was added to each sample and incubated for 20 min at room temperature. Next, 3.15 M perchloric acid and 20 % p-dimethylaminobenzaldehyde were added and samples were incubated for additional 20 min at 60 °C. The absorbance was determined at 557 nm with a Spectra MAX 190 microplate spectrophotometer.

Immunohistochemistry

Formalin-fixed, paraffin-embedded skin sections were stained with anti-JunD antibodies (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) or anti-SMA (clone 1A4, Sigma-Aldrich, Steinheim, Germany) antibodies. Peroxidase labeled species-specific immunglobulins (Dako, Glostrup, Denmark) were used as secondary antibodies. Irrelevant isotype matched antibodies were used as controls. Staining was visualized with DAB

peroxidase substrate solution (Sigma-Aldrich). αSMA stained sections were counterstained with hematoxylin. The number of myofibroblasts was determined at 200-fold magnification. Counting was performed in a blinded manner by two independent examiners.

Quantitative real time-PCR

Gene expression was quantified by SYBR Green real time-PCR using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described [21]. The following primer pairs were used: human JunD: 5'-AAGAGTCAGAACACGGAGCTG-3' (forward), 5'-GGCTGAGGACTTTCTGCTTG-3' (reverse); human Smad3: 5'-GCTGGGCTGAAGCGCACTGA-3' (forward), 5'-GGCTGCGAGGCGTGGAATGT-3' (reverse); human Smad4: 5'-GCCACCCAAACCGCTCCGT-3' (forward), 5'-CACGGCCCTGGTCGTCGTC-3' (reverse); murine JunD: 5'-ACTACCCCGACCAGTACGC-3' (forward), 5'-TCGCTAGCTGCCACCTTC-3' (reverse); murine col1a1: 5'-GAAGCACGTCTGGTTTGGA-3' (forward), 5'-ACTCGAACGGGAATCCATC-3' (reverse); murine col1a2: 5'-TCAAACTGGCTGCCACCAT-3' (forward), 5'-CCAACAAGCATGTCTGGTTAGGA-3' (reverse); murine PAI -1: 5'-ACGTTGTGGAACTGCCCTAC-3' (forward), 5'-AGCGATGAACATGCTGAGG-3' (reverse); murine Smad7: 5'-GCTCAATTCGGACAACAAGAG-3' (forward), 5'-TCTTGCTCCGCACTTTCTG-3' (reverse); Samples without enzyme in the reverse transcription reaction (Non-RT controls) were used as negative controls. Unspecific signals caused by primer dimers were excluded by no template controls and by dissociation curve analysis. β-actin was used to normalize for the amounts of cDNA within each sample.

Statistics

Data were expressed as mean ± standard error of the mean. The student's t-test was used for statistical analyses. A p-value of less than 0.05 was considered statistically significant.

RESULTS

JunD is overexpressed in SSc patients

To analyze the expression of JunD in SSc patients, we first determined the mRNA levels of JunD in skin biopsies from SSc patients and healthy volunteers. The mRNA levels of JunD were increased by 3.1 ± 0.3 fold in clinically involved skin of SSc patients compared to controls ($p = 0.04$) (Figure 1a). A comparable upregulation of JunD mRNA was observed in clinically non-involved skin (3.1 ± 0.3 fold). An upregulation of JunD in SSc patients was also observed by immunohistochemistry of the dermis. A widespread expression of JunD was observed in skin sections from clinically involved skin of SSc patients with a prominent staining in fibroblasts and endothelial cells, but also in keratinocytes (Figure 1b). The expression pattern in clinically non-involved skin was identical to that in involved skin. In contrast to SSc patients, the expression of JunD was largely restricted to keratinocytes in healthy individuals and either absent or very weak in fibroblasts and endothelial cells.

Increased levels of JunD were also observed in cultured fibroblasts of SSc patients. The mRNA levels of JunD were significantly increased in SSc by 2.2 ± 0.3 fold compared to control fibroblasts ($p = 0.03$) (Figure 1c). Consistently, the protein levels of JunD were also upregulated in SSc fibroblasts by 1.8 ± 0.1 fold ($p = 0.01$) (Figure 1d).

TGF-β stimulates the expression of JunD in a Smad dependent manner

We hypothesized that the upregulation of JunD in SSc fibroblasts might be mediated by TGF-β. Indeed, incubation of fibroblasts with TGF-β stimulated the expression of JunD and induced nuclear accumulation of JunD. The expression of JunD mRNA and protein increased within 1 h after stimulation with TGF-β and the protein levels remained elevated for 24 h (Figures 2a and 2b). JunD was located in the cytoplasm in resting cells and within the first 3 h after stimulation, but prominently accumulated in the nucleus between 6 and 9 h after stimulation with TGF-β (Figure 2b).

To determine whether TGF-β induces JunD via the canonical Smad pathway, the expression of Smad 3 and Smad 4 was targeted by siRNA in human fibroblasts. siRNA against Smad 3 and Smad 4 reduced the mRNA and protein levels of the respective proteins by > 85 % (data not shown). Knockdown of Smad 3 almost completely prevented the induction of JunD by TGF-β with a decrease of 80 ± 14 % ($p = 0.04$) compared to fibroblasts transfected with mock siRNA (Figure 2c). siRNA against Smad 4 also prevented the upregulation of JunD by TGF-β (data not shown), demonstrating that the canonical Smad cascade is essential for the induction of JunD by TGF-β.

To confirm the central roles of TGF-β and Smad signalling for the induction of JunD in SSc, SSc sections were stained for phosphorylated and thus activated Smad 3 (pSmad 3) and JunD. Double staining revealed co-localisation of JunD with pSmad3 in > 90 % of JunD positive cells. Vice versa, virtually all cells positive for pSmad 3 expressed high levels of JunD (Figure 2d).

Lack of JunD reduces the stimulatory effects of TGF-β on collagen synthesis

We next investigated, whether the induction of JunD might contribute to the pro-fibrotic effects of TGF-β. We first analyzed, whether deficiency of JunD might decrease the expression of classical TGF-β target genes in fibroblasts. Indeed, the stimulatory effects of TGF-β on the mRNA levels of CTGF were decreased by 80 ± 11 % in $JunD^{-/-}$ fibroblasts compared to $JunD^{+/+}$ cells ($p = 0.02$) (Figure 3a). The induction of PAI-1 and Smad7 were also decreased by 75 ± 23 % and 88 ± 22 % in $JunD^{-/-}$ fibroblasts ($p = 0.01$ for PAI-1 and $p = 0.04$ for Smad7) (Figures 3b and 3c) demonstrating a decreased responsiveness of $JunD^{-/-}$ fibroblasts towards TGF-β.

The upregulation of type I collagens upon stimulation with TGF-β was also reduced in $JunD^{-/-}$ fibroblasts. The induction of col 1a1 and col 1a2 were decreased by 46 ± 15 % and 70 ± 7 % in $JunD^{-/-}$ fibroblasts compared to $JunD^{+/+}$ fibroblasts ($p = 0.04$ for col 1a1 and $p = 0.03$

for col 1a2) (Figures 3d and 3e). Similar findings were also obtained on the protein level with a significant reduction of the TGF-β induced hydroxyproline content in $JunD^{-/-}$ by 83 ± 15 % ($p = 0.03$) (Figure 3f). Consistent with the low expression of JunD in resting fibroblasts, the basal collagen synthesis was not affected in $JunD^{-/-}$ fibroblasts.

Mice deficient for JunD have ameliorated bleomycin induced dermal fibrosis

To study the role of JunD in experimental fibrosis, the mouse model of bleomycin induced dermal fibrosis was used. Similar to the findings in SSc patients, an increased expression of JunD was also observed in wildtype mice challenged with bleomycin (Figure 4a), confirming that the model of bleomycin induced fibrosis is suitable to study the role of JunD.

No differences in skin histology were observed between $JunD^{-/-}$ mice and $JunD^{+/+}$ mice in the absence of a fibrotic stimulus (Figure 4b). Injections of bleomycin induced prominent fibrosis in $JunD^{+/+}$ with increased dermal thickness, activation of fibroblasts and accumulation of collagen (Figures 4c-e). The fibrotic effects to bleomycin were significantly ameliorated in $JunD^{-/-}$ mice. Dermal thickening in lesional skin was reduced by 53 ± 8 % in $JunD^{-/-}$ mice compared to $JunD^{+/+}$ mice ($p = 0.002$) (Figures 4c). The number of myofibroblasts and the hydroxyproline content in lesional skin were also significantly reduced by 64 ± 9 % and 84 ± 6 %, respectively ($p = 0.0001$ for the number of myofibroblasts and $p = 0.003$ for the hydroxyproline content) (Figures 4d and 4e). All outcomes remain statistically significant after Bonferroni correction for multiple testing.

DISCUSSION

The present study demonstrates a role of JunD as a downstream mediator of the pro-fibrotic effects of TGF-β in SSc fibroblasts. (i) JunD is induced by TGF-β within 1 hour and its expression co-localizes with phosphorylated Smad3 as a marker for active TGF-β signalling in vivo. (ii) The expression of classical TGF-β target genes such as PAI-1, Smad7 and CTGF is prevented in fibroblasts deficient for JunD. (iii) Deficiency for JunD abrogates the TGF-β induced differentiation of resting fibroblasts into myofibroblasts. (iv) The stimulatory effects of TGF-β on collagen synthesis are reduced in fibroblasts lacking JunD. (v) JunD also regulates the expression of other pro-fibrotic mediators such as interleukin-6 and tissue inhibitor of matrix metallo-proteinases-1 (TIMP-1) [22-24]. (vi) $JunD^{-/-}$ mice are protected from bleomycin induced experimental fibrosis. These findings may have direct translational implications. The mild phenotype of mice completely deficient for JunD [9] suggests that pharmacologic targeting of JunD may not be prevented by toxicity. Although specific inhibitors for JunD are currently not available, small molecule inhibitors of AP-1 signalling have recently been described and are currently tested in clinical trials [25]. However, further studies are needed to evaluate JunD as a potential target for anti-fibrotic therapies. In particular, the role of JunD for fibrosis should be confirmed in additional, less inflammatory mouse models of SSc.

We demonstrated that JunD is overexpressed in fibrotic skin of SSc patients and that this overexpression is induced by TGF-β via activation of canonical Smad signalling. Increased levels of JunD were also detected in cultured SSc fibroblasts, consistent with the characteristic autocrine activation of TGF-β in SSc fibroblasts in vitro [26-27]. Considering the role of JunD for the stimulatory effects of TGF-β on fibroblast activation, the upregulation of JunD in all SSc lines suggests that the persistent activation of JunD in SSc fibroblasts might contribute to the characteristic activated phenotype of SSc fibroblasts with persistently increased expression of myofibroblast markers and collagen synthesis even in the absence of

exogenous stimuli and after several passages in culture. Interestingly, we did not observe differences in the expression levels of JunD between patients positive and negative for anti-topoisomerase or anti-centromer autoantibodies, suggesting that the status of conventional autoantibodies is not related to the expression of JunD.

Although it was beyond the scope of our study, JunD might also contribute to vascular disease in SSc by functioning as a dimerisation partner of the Fos-related antigen-2 (Fra-2). Fra-2 is overexpressed in vessels of SSc patients and transgenic overexpression of Fra-2 in mice results not only in proliferation of pulmonary vascular smooth muscle cells and in histological changes resembling those of pulmonary arterial hypertension in humans, but also in a progressive microangiopathy with capillary rarefication [28-29]. Fra-2 dimerizes with members of the Jun family of AP-1 transcription factors to regulate the expression of target genes. JunD seems to be the preferred dimerisation partner in the context of TGF-β signalling as evidenced by studies on the laminin α3 gene [30]. The hypothesis of a role of JunD / Fra-2 dimers in SSc vasculopathy is further supported by the expression patterns of both genes in SSc patients. The expression pattern of JunD was identical to that of Fra-2 and included a prominent expression of JunD and Fra-2 in endothelial cells [31]. However, further studies are needed to address a potential role of JunD in the vascular manifestations of SSc.

In summary, we identify JunD as mediator of TGF-β signalling in SSc. JunD is overexpressed in a TGF-β / Smad dependent manner in SSc and mediates the pro-fibrotic effects of TGF-β on SSc fibroblasts. Deficiency of JunD reduces the activation of fibroblasts and prevents experimental fibrosis. These findings might have translational implications, because inhibitors of AP-1 signalling have been developed and would be available for clinical trials in SSc. However, further studies are needed to evaluate JunD as a potential therapeutic target in SSc.

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COMPETING INTERESTS

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1: JunD is overexpressed in patients with SSc.

Figure 1a: The mRNA levels of JunD were elevated in the skin of SSc patients compared to healthy controls. **Figure 1b:** A prominent staining for JunD protein was observed by immunohistochemistry in fibroblasts, keratinocytes and endothelial cells of SSc patients. In contrast, the expression of JunD was significantly reduced in age- and sex matched healthy individuals (n = 7 for both). Two representative overview sections and a negative (isotype) control are shown at 200 fold magnification. In addition, a staining from a SSc patient is shown at 1000 fold magnification to show the intense staining in fibroblasts and vessels. The epidermis is marked with E, the dermis with D. Some representative fibroblasts are marked with arrows, vessels with arrow heads.

Figures 1c and 1d: The overexpression of JunD in fibroblasts persisted in vitro. Increased mRNA (**Figure 1c**) and protein levels (**Figure 1d**) of JunD were detected by real-time PCR and Western Blot in SSc fibroblasts compared to control cells.

* indicates statistical significant differences as compared to healthy volunteers and control fibroblasts. V = vessels, F = spindle shaped fibroblasts

Figure 2: TGF-β induces JunD in a Smad dependent manner.

Figure 2a: Stimulation with TGF-β induced the mRNA levels of JunD in healthy dermal fibroblasts a time-dependent manner with a maximal induction after 1 h. **Figure 2b:** TGF-β increased the protein levels of JunD and induced nuclear translocation in healthy dermal fibroblasts as analyzed by immunofluorescence. The expression of JunD was increased after stimulation with TGF-β for 1 h and translocated into the nuclei after 6 h. The expression of JunD protein was visualized with FITC labeled secondary antibodies (green staining) and nuclei of fibroblasts were stained with DAPI (blue staining).

Figure 2c: Transfection of fibroblasts from healthy volunteers with siRNA against Smad3 prevented the induction of JunD by TGF-β. **Figure 2d**: Phosphorylated and thereby activated Smad3 (red staining) and JunD (brown staining) co-localize in skin biopsies of SSc patients as detected by immunohistochemistry. Representative tissue sections are shown at 200 fold (upper images), a detailed view of JunD and pSmad3 double positive stained cells is shown in SSc sections at 1000 fold magnification (lower images).

* indicates statistical significant differences as compared to unstimulated fibroblasts (Figures 2a and 2b) or compared to fibroblasts transfected with non-targeting siRNA (Figure 2c).

Figure 3: Deficiency of JunD reduces the responsiveness towards TGF-β.

Figures 3 a-c: The induction of typical TGF-β target genes such as CTGF (**Figure 3a**), PAI-1 (**Figure 3b**) and Smad7 (**Figure 3c**) was reduced in fibroblasts deficient for JunD compared to controls.

Figures 3 d-f: The stimulatory effects of TGF-β on the mRNA levels of col 1a1 (**Figure 3d**), col 1a2 (**Figure 3e**) as well as the increased release of hydroxyproline (**Figure 3f**) were also decreased in $JunD^{-/-}$ fibroblasts compared to controls.

* indicates statistical significant differences as compared to wildtype cells.

Figure 4: JunD is crucial for the development of experimental fibrosis.

Figure 4a: The expression of JunD was increased in the mouse model of bleomycin induced skin fibrosis. Representative images of control mice injected with NaCl, of mice challenged with bleomycin and of a control section incubated with isotype control antibodies are shown at 200 fold magnification. **Figure 4b:** Reduced dermal thickening in mice lacking JunD. Representative tissue sections at 100 fold magnification are shown: $JunD^{+/+}$ mice injected with NaCl intracutaneously (n = 7), $JunD^{-/-}$ mice with intracutaneous injections of NaCl (n = 8), bleomycin-injected $JunD^{+/+}$ mice (n = 7), $JunD^{-/-}$ mice injected with bleomycin (n = 6).

Figure 4c: Deficiency of JunD prevented the differentiation of resting fibroblasts into myofibroblasts upon challenge with bleomycin. **Figure 4d:** Decreased collagen content in lesional skin of bleomycin-challenged $JunD^{-/-}$ mice compared to wildtype littermates. In total, 20 $JunD^{-/-}$ mice and 16 $JunD^{+/+}$ littermates were analyzed.

* indicates statistical significant differences compared to bleomycin-injected $JunD^{+/+}$ mice.